Effect of cyclic stretch on $\beta_{1D}$-integrin expression and activation of FAK and RhoA

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Zhang SJ, Truskey GA, Kraus WE. Effect of cyclic stretch on $\beta_{1D}$-integrin expression and activation of FAK and RhoA. Am J Physiol Cell Physiol 292: C2057–C2069, 2007. First published January 31, 2007; doi:10.1152/ajpcell.00493.2006.—Integrins play a pivotal role in proliferation, differentiation, and survival in skeletal and cardiac myocytes. The $\beta_{1D}$-isoform of the $\beta_1$-integrin is specifically expressed in striated skeletal muscle. However, little is known about the role and the mechanisms by which the splice variant $\beta_{1D}$-integrin regulates myogenesis and mechanotransduction. We observed that cyclic mechanical stretch increases $\beta_{1D}$-integrin protein levels and activates the downstream cytoskeletal signaling proteins focal adhesion kinase (FAK) and RhoA. Elimination of native $\beta_{1D}$-integrin expression by RNA interference in immature developing myoblasts abolished stretch-induced increases in FAK phosphorylation and further downregulated RhoA activity. Blocking of $\beta_{1D}$-integrin expression prevented myoblastic fusion to form multinucleated mature myotubes. Restoration of human $\beta_{1D}$-integrin expression in $\beta_{1D}$-integrin-deficient cells partially restored myotube formation. The onset of myofusion also requires the generation of nitric oxide (NO). The release of NO affects cytoskeletal proteins by mediating RhoA activity and protein degradation. Our previous study demonstrated that stretch-induced NO positively modulates mechanical properties of differentiating skeletal myocytes. We found a significant decrease in NO production and apparent elastic modulus in $\beta_{1D}$-integrin-deficient cells, suggesting signaling interactions between $\beta_{1D}$-integrin and neuronal NO synthase to mediate mechanotransduction and myogenesis in skeletal myocytes. These results suggest that, in addition to regulating differentiation, the $\beta_{1D}$-integrin isoform plays a critical role in the response of skeletal myoblasts to cyclic stretch by activating the downstream components of FAK and RhoA activity and affecting NO release.

focal adhesion kinase; RhoA activity; mechanotransduction; skeletal myocytes

MECHANICAL STIMULI PLAY an important role in embryonic and neonatal muscle development. On a cellular level, mechanical stretch influences a number of important events, such as cell proliferation, differentiation, metabolism, remodeling, repair, and survival (6, 12, 19, 21, 30). For instance, the continuous elongation of bones during embryonic development results in activation of skeletal progenitor cells, known as myoblasts, and subsequent differentiation of myoblasts into mature muscle cells (16). Passive stretch of mature muscle leads to an increase in muscle tension that, in turn, promotes skeletal muscle growth by augmenting protein synthesis and accumulation of muscle mass (1).

Passive stretch activates integrin-mediated signaling pathways (18, 26, 31). The activation of integrins is essential for maintaining the normal biological functions of skeletal muscle cells, such as cell proliferation, differentiation, and survival (32, 33). The integrin $\beta_1$-subunit is the major $\beta$-integrin expressed in skeletal muscle (24). Two splice variants of the $\beta_1$-integrin subunit, $\beta_{1A}$ and $\beta_{1D}$, are developmentally regulated in skeletal myocytes (38). $\beta_{1A}$-integrin is highly expressed in proliferating myoblasts, is downregulated during myodifferentiation, and is mainly associated with the $\alpha_5$-subunit (33). The majority of $\beta_{1D}$-integrin binds to $\alpha_7$-integrin in developing mononuclear myoblasts as they fuse to form maturing multinucleated skeletal myocytes, or myotubes (4). The switch in the differential splicing of $\beta_1$-integrin from $\beta_{1A}$ to $\beta_{1D}$ reflects their distinct roles in the different stages of skeletal myogenesis (4, 22, 44). This notion is supported by an in vivo study that showed impaired fusion and defective cytoskeleton in these $\beta_{1D}$-integrin-deficient myoblasts (35). Thus it is conceivable that the expression of $\beta_{1D}$-integrin is required to strengthen the linkage between cytoskeleton and extracellular matrix in the developing myotube. Localization of $\beta_{1D}$-integrin at the sarcolemma in skeletal myocytes may enable it optimally to sense changes in the mechanical environment, where it serves an important role in sensing and transducing mechanical signals from the external environment to the cytoplasm (4).

Integrin-mediated signaling involves downstream activation of associated adaptor proteins, such as focal adhesion kinase (FAK), and small Rho GTPases (18, 34, 36, 37). In various cell types, it has been shown that on integrin binding to ligand and subsequent clustering of integrins, FAK becomes autophosphorylated, mainly at the site of Tyr397, to form Src/FAK signaling complexes (34, 43). These signaling complexes are key to activation of downstream components that, in turn, form focal adhesion complexes. Modulation of phosphorylation of FAK at Tyr397 affects myoblast differentiation (11). $\beta_{1D}$-integrin may also interact with Rho GTPases. In fibroblasts activation of Rho GTPases is induced by the assembly of focal complexes, and the activity of Rho GTPases is critical for integrin clustering and partial phosphorylation of FAK (9, 10, 13, 17). Similar to FAK, Rho GTPases play an important role in myodifferentiation. For instance, an active form of RhoA is required for the expression of a myogenic transcription factor and a key differentiation regulator, serum response factor (SRF) and MyoD (7, 40). These Rho family proteins also regulate the organization of the actin cytoskeleton.

Nitric oxide (NO), a short-lived second messenger, also plays an important role in mechanotransduction and myoblast fusion (28, 41, 42). Our previous work (47) showed that NO is sensitive to mechanical stretch and it influences mechanical
properties of differentiating myoblasts. A study by Zuckerbraun et al. (48) found that the release of NO in smooth muscle cells affects RhoA activity. Thus it is likely that β1D-integrin interacts with neuronal NO synthase (nNOS) through RhoA. While β1D-integrin, FAK, RhoA GTPases, and nNOS each affect skeletal myogenesis (11, 35, 40), the effects of mechanical stimulation on β1D-integrin levels and the subsequent activation of FAK and RhoA in skeletal myocytes are not well known. In this study, we tested the hypotheses that cyclic mechanical stretch induces the expression of β1D-integrin, which, in turn, influences NO production and the downstream signaling proteins of integrin pathway, FAK and RhoA, and that β1D-integrin is a key mediator of mechanotransduction during skeletal muscle differentiation.

MATERIALS AND METHODS

β1D-integrin hairpin siRNA design. We designed the short hairpin small interfering (si)RNA (shRNA) to interfere with β1D-integrin expression, according to the parameters indicated on the siRNA Target Finder website (http://www.dharmacon.com and http://www.ambion.com). The design of shRNA target sites for stable RNA interference (RNAi) transfection and transient siRNA is similar. The forward oligonucleotides are 5'-GATCCTCCAAACTATGGACGTAAAT- TCAAGAGATTTACGTCCATAGTTTGGATTA-3', and the reverse oligonucleotides are 3'-GAGGTTTGATACCTGCATTTAAGT- TCTCTAAATGCAGGTATCAAACCTAATTCGA-5'. Transient siRNA with the same target sequence (5'-AATCCAAACTATGGACGTAAAT- TCAAGAGATTTACGTCCATAGTTTGGATTA-3'; Dharmacon) was tested first to ensure the knockdown efficiency in C2C12 cells. Western blotting and immunofluores-

Fig. 1. Effects of cyclic stretch on actin cytoskeleton, differentiation and hypertrophy, and β1D-integrin protein expression. A: cyclic stretch induces actin fiber formation. To assess the effects on actin cytoskeleton, cells were subjected to 10% cyclic stretch at 0.5 Hz with 1 h on and 5 h off for up to 4 days and fixed on day 4 and double-stained with rhodamine phalloidin and Sytox Green for nuclei. All stretch conditions were the same unless otherwise noted. B: cyclic stretch induces maturation of C2C12 cells. To evaluate effects of cyclic stretch on differentiation, after 4 days of stretch cells were fixed and stained with monoclonal anti-α-actinin and Sytox Green to stain nuclei. White arrows point to Z lines. C: β1D-integrin is upregulated in response to stretch. Cells were extracted after 2 days and 4 days of cyclic stretch, and Western blot analysis was performed and normalized by the protein levels of unstretched cells on day 2. C.1: representative Western blot of β1D-integrin in response to 4 days of cyclic stretch. C.2: normalized values of protein level of β1D-integrin in response to 4 days of cyclic stretch. Values represent means (SD), and n = 4 separate experiments. **P < 0.01, ***P < 0.001 compared with static group. D: immunostaining of β1D-integrin in differentiating C2C12 cells. Cells were stained with monoclonal anti-β1D-integrin antibody and Sytox Green for nuclei on days 2 and 4, respectively.
cence were used to validate blocking of integrin expression. Control cells were treated with a scrambled shRNA sequence.

**Cell culture.** Murine C2C12 myoblasts (American Type Culture Collection), a subclone cell line, were grown on a six-well tissue culture plate (Flexcell International) coated with 100 μg/ml growth factor-reduced Matrigel (GFR Matrigel, BD Biosciences). Early-passage cells (passages 3–4) were used in all experiments to avoid dedifferentiation in C2C12 cells. The cells were fed daily with growth medium (GM) containing Dulbecco’s modified Eagle’s medium (DMEM; GIBCO BRL), 10% newborn calf serum (HyClone Laboratories), 0.5% chicken embryo extract (GIBCO BRL), and 0.5% gentamicin (GIBCO BRL) and incubated at 37°C with 95% air-5% CO2 until the cells reached 90–95% confluence. Subsequently, the GM was shifted to the differentiation medium (DM), consisting of DMEM supplemented with 10% horse serum (Intergen) and 0.5% gentamicin to promote differentiation of myoblasts to fuse into myotubes.

**Generation of β1D-integrin-deficient C2C12 cells and rescue experiment.** C2C12 myoblasts at 60–70% confluence were divided into two groups and transfected with 2 μg of purified DNA plasmid with TransIT-LT1 transfection reagent (Mirus), according to the manufacturer’s instruction. To obtain stable C2C12 transfectants, the cells were selected in GM containing geneticin (G418, 400 μg/ml; GIBCO). When the transfected cells were confluent, they were maintained in DM with 400 μg/ml geneticin. A circular pSilencer 4.1-CMV neo vector (Ambion) with a scrambled shRNA was used to control for...
nonspecific effects in all experiments. Cell extracts were collected and subjected to 4–12% Bis-Tris SDS-PAGE (Invitrogen) and Western blotting with an anti-β1D-integrin monoclonal antibody (Chemicon). Cell extracts were collected and subjected to SDS-PAGE and Western blotting with β1D-integrin monoclonal antibody. Clones with the best-inducible inhibition of β1D-integrin were selected for further experiments.

Repression of β1D-integrin by stably transfected shRNA was recovered by expressing human cDNA β1D-integrin vector, a generous gift from Dr. Arnoud Sonnenberg (Netherlands Cancer Institute, Amsterdam, The Netherlands). The construction of this vector was described previously (15, 45). Similarly, the vector was introduced into β1D-integrin-inhibited cells with Trans-LT1 (Mirus), according to the manufacturer’s instructions. After transfection, cell lysates were extracted and subjected to SDS-PAGE, and Western blotting was performed to confirm the reexpression of β1D-integrin protein.

Cell stretching. After cells reached 90% confluence, the medium was changed to the low-growth factor DM. A mechanical stretch device (Flexcell International) was used to apply the desired mechanical stimuli. After cells became confluent, C2C12 myocytes or β1D-integrin-deficient cells were subjected to cyclic equibiaxial stretch consisting of 10% elongation at 0.5 Hz with 1 h on and 5 h off for 0–4 days. Cells on days 2 and 4 were collected or fixed for further experimental procedures.

Inhibition of RhoA activity with Clostridium botulinum C3 ADP-ribosyltransferase. C2C12 myocytes were plated at a density of 1 × 10^5 cells per well in a six-well plate and cultured in GM at 37°C and 5% CO2. When cells were 60–70% confluent, the inhibitor of RhoA C. botulinum C3 transferase (Upstate) was transected into cells with protein transfection reagent Chariot (Active Motif), according to manufacturer’s instructions. Transfected cells were maintained in GM for 24 h to become confluent before change to DM.

Immunostaining. C2C12 myocytes were fixed with 3.7% formaldehyde and permeabilized in 1% NP-40 (Sigma) in PBS for 15 min. Cells were then incubated with 10% horse serum in PBS for 1 h to block nonspecific binding. Cells were immunostained with either anti-β1D-integrin (Chemicon) or anti-α-actinin (Sigma) (1:200) in PBS for 1 h. The primary antibody was detected with a secondary mouse anti-IgG conjugated with rhodamine secondary antibody (Molecular Probes). Sytox Green (Molecular Probes) was added with the secondary antibody as a counterstain for nuclei (Molecular Probes), according to the manufacturer’s manual. To assess the effect of mechanical stretch and/or knockdown of β1D-integrin on cytoskeleton of differentiating C2C12 myocytes fixed and permeabilized, cells were incubated with phalloidin (Molecular Probes) and Sytox (Molecular Probes), according to manufacturer’s instructions.

Antibodies and immunoprecipitation. Western blot and immunoprecipitation were carried out as described previously (29, 47). Antibodies to β1D-integrin (Chemicon), FAK (Upstate), FAK Tyr397 (Upstate), RhoA (Upstate), and RhoA-GTP (Upstate) were used to detect proteins and immunoprecipitation. Mouse IgG horseradish peroxidase-conjugated antibody was purchased from Santa Cruz. Protein levels were quantified by densitometry and normalized to the unstretched wild-type cells on day 2 to minimize variability between experiments. Three independent experiments were used for each condition.

RhoA pulldown assay. Activated RhoA GTPase was determined with a RhoA activation assay kit (Upstate), according to the manufacturer’s instructions. Briefly, at 48 h and 96 h after switch to DM, cells were washed in ice-cold Tris-buffered saline and lysed in lysis buffer provided by the manufacturer. The lysates were centrifuged for 5 min at 14,000 g at 4°C and were immediately incubated with Rhotekin RBD-agarose for 45 min at 4°C with agitation. The beads were collected by centrifugation and washed with Mg2⁺ lysis/wash buffer three times, and the pellets were eluted with 40 μl of 2× SDS sample buffer. Samples were separated by 4–12% Bis-Tris gel (Invitrogen), followed by Western blotting with anti-RhoA antibody (Upstate).

Bromodeoxyuridine incorporation. Cell replication was quantified by bromodeoxyuridine (BrdU) incorporation. BrdU was added to cell cultures at either 47 h or 95 h after switch to DM, and cells were incubated for 1 h at 37°C and 5% CO2. At the end of the incubation BrdU was removed and the cells were rinsed. Cells were fixed and denatured with fixative/denaturing solution from the manufacturer (Roche Applied Bioscience), and the BrdU assay was performed according to the manufacturer’s instructions.

NO assay and cell elastic modulus. NO production was determined as previously described (47). Atomic force microscopy was performed as previously described (47). The apparent elastic modulus of the cells was obtained from force-indentation curves with the Hertz equation to calculate for a conical tip as shown below:

\[ F = k \cdot d = E \cdot \delta \cdot (z - d) \]  

(1)

where \( F \) is the applied loading force, \( k \) is the spring constant of the AFM cantilever, \( d \) is the deflection of the cantilever, \( E \) is the elastic modulus of the sample, \( \delta \) is the indentation depth, \( z \) is the piezo height, and \( \Omega \) is the function of the cantilever geometry. For the conical tip we used, \( \Omega \) is:

\[ \Omega = \frac{2 \cdot \tan(\alpha)}{\pi \cdot (1 - v^2)} \]  

(2)

where \( \alpha = 35° \) is the angle of the cone tip (Digital Instruments) and \( v \) is the Poisson ratio. A value of \( v = 0.5 \) was used. The elastic modulus was determined by calculating the slope of force vs. the square of the indentation from the raw data.

Statistical analysis. All values are expressed as means (SD) unless otherwise noted. Student’s t-test or two-way ANOVA was performed with GraphPad Prism version 4.00 (GraphPad). Bonferroni post hoc test was applied for the two-way ANOVA analysis to determine the significance of differences between groups. A \( P \) value of <0.05 was considered significant.

RESULTS

Upregulation of β1D-integrin in response to stretch is associated with enhanced myotube formation. To determine whether cyclic cellular deformation through mechanical stretch affects skeletal myotube formation, differentiating myoblasts were subjected to repeating patterns of 10% equibiaxial cyclic stretch at 0.5 Hz for 1 h followed by 5 h of rest for up to 4 days. Cells were either extracted for protein analysis or fixed and stained with rhodamine-conjugated phalloidin or α-actinin and counterstained with Sytox Green to visualize nuclei. Cyclic stretch for 4 days induced prominent stress fiber formation (Fig. 1A) and Z line formation (Fig. 1B). The Z line formation is indicative of mature skeletal myocytes. Z line formation was observed in 69% (SD 25%) cells under stretch, compared with 41% (SD 13%) cells under static conditions (\( P < 0.001 \)) (Table 1). Furthermore, cyclic stretch produced significantly larger myotube diameter (Table 1). These data suggest that stretch enhances myotube formation and differentiation.

To examine whether enhanced cross-striation of α-actinin staining, organized actin stress fibers, and induced myotube formation are associated with increases in the protein level of β1D-integrin, cell extracts were subjected to SDS-PAGE and immunoblotting was performed to measure β1D-integrin levels after cyclic stretch for 2 or 4 days (Fig. 1C). After 2 days of stretch, β1D-integrin protein was significantly upregulated by up to eight times compared with unstretched cells. As myoblasts differentiated and fused to form myotubes, the level of...
β1D-integrin also increased in unstretched cells. After 4 days of cyclic stretch β1D-integrin protein levels were approximately three times higher than the levels found with unstretched cells. The significant increase of β1D-integrin expression level on day 2 coincided with the formation of myotubes, consistent with the role of β1D-integrin in the induction of myocyte differentiation.

To further confirm that the expression of β1D-integrin is associated with the onset of myotube formation, cells were immunostained for β1D-integrin after stretch on days 2 and 4. β1D-integrin was expressed in differentiating myoblasts and highly expressed in myotubes (Fig. 1D). Cyclic stretch induced myotube formation, which was associated with pronounced increases of β1D-integrin. The observed increases of β1D-integrin by immunostaining in unstretched as well as in stretched cells from days 2–4 are correlated with the change of protein levels of β1D-integrin as quantified on Western blots.

Cyclic stretch activates a β1D-integrin-mediated signaling pathway. To assess whether mechanical stretch activates β1D-integrin-mediated signaling pathways, the downstream components of FAK and Rho GTPases were examined. Relative to unstretched cells, total FAK protein levels were unchanged in stretched cells relative to control cells (Fig. 2A), which is consistent with other reports (11, 21). However, phosphorylation of tyrosine at 397 was markedly upregulated by up to two times in response to stretch (Fig. 2B). Interestingly, the expression level of FAK Tyr397 decreased from day 2 to day 4 in both unstretched and stretched cells (Fig. 2E). The relative decrease in FAK Tyr397 from day 2 to day 4 was more pronounced in unstretched cells versus stretched cells.

As with total FAK, the total RhoA level was not significantly altered by stretch (Fig. 2C). The active form of RhoA, examined in a Rho immunoprecipitation assay, was significantly increased by cyclic stretch, from 5.8 times (SD 0.96) greater than the level found with unstretched cells on day 2 to 3.5 times (SD 0.35) on day 4 (Fig. 2D). The increase in RhoA activity induced by stretch appears to be associated with the increase in β1D-integrin. Together, the data imply that the commitment to myogenesis appears to be coordinated with the upregulation of cellular β1D-integrin protein content, FAK phosphorylation, and RhoA activity and that the expression of β1D-integrin enhances myodifferentiation by regulating FAK and RhoA.

Establishment of β1D-integrin-deficient C2C12 cells with siRNA. To confirm the central role of β1D-integrin in myogenesis and mechanotransduction, we used a DNA vector including a shRNA in stable transfection experiments to inhibit the expression of β1D-integrin. shRNA treatment specific to β1D-integrin suppressed differentiation in C2C12 cells (Fig. 3A, I). Protein from cells transfected with shRNA to β1D-integrin and control cells transfected with scrambled shRNA was extracted on days 2 and 4 and subjected to SDS-PAGE and Western blotting. β1D-integrin protein levels were decreased by ~90% on day 4 (Fig. 3A,2) relative to negative control cells. β1D-integrin protein levels were not significantly different in negative control cells receiving the shRNA scrambled sequence compared with wild-type cells (Fig. 3A,3). Furthermore, when these β1D-integrin-deficient cells were subjected to mechanical stretch for up to 4 days, β1D-integrin protein levels, indicated by immunofluorescence, were unchanged (Fig. 3B). Notably, no adjacent and aligned nuclei were observed, indicating that no fusion occurred in these cells. These data suggest that β1D-integrin functions as a mechanical transducer to induce differentiation in response to mechanical stimuli and is required to activate skeletal myogenesis pathways.

Since overexpression of β1D-integrin fails to activate mitogen-activated protein kinases (MAPK) and inhibits cell growth in C2C12 cells (2), we expected that suppressing β1D-integrin would inhibit cells from exiting the cell cycle and promote continued cellular proliferation despite cellular cues toward myocyte differentiation. To confirm this, we used a BrdU incorporation assay to assess continued nuclear DNA synthesis after exposure of cultures to myocyte differentiation medium that is deprived of cellular growth factors and promotes cellular differentiation. We observed that ~35% (SD 5.98%) of the total number of cells remained BrdU positive under these conditions. By day 4, the number of BrdU-positive cells further declined to 14% (SD 6.67%) (Fig. 3C). Cyclic stretch led to a significant reduction in BrdU-positive cells to 19% (SD 7.09%) on day 2 and 7% (SD 2.16%) on day 4, suggesting that most of the cells were committed to a myocyte differentiation pathway under these conditions. Induction of scrambled shRNA did not cause significant changes in BrdU incorporation in the negative control cells compared with wild-type cells under the same conditions. In contrast, inhibition of β1D-integrin led to 89% (SD 4.0%) of BrdU-positive cells on day 2. Even by day 4, ~40% (SD 6.36%) of the cells were still BrdU positive, suggesting that suppression of β1D-integrin expression maintained the vast majority of cells in a proliferation mode. Stretch was not able to overcome the cellular block to differentiation in the absence of β1D-integrin, and the number of BrdU-positive cells was significantly higher under these conditions than in static and stretched wild-type cells. Together, the BrdU data indicate that β1D-integrin is a key protein in mediating normal myocyte differentiation, likely by regulating key cellular events in myoblast fusion.

Reexpression of β1D-integrin expression restores myotube formation. To further demonstrate the specificity of β1D-integrin-mediated effects on myocyte mechanotransduction and myogenesis, β1D-integrin expression was restored in transfection experiments and the results assessed. The human β1D-integrin cDNA construct as earlier described (45), which contains a single nucleotide difference in the targeting region for the endogenous murine RNAi and thus would not present an effective target for constitutively expressed anti-murine RNAi, was introduced in the β1D-integrin-repressed cells. Human-specific β1D-integrin expression was detectable after 4
days, the protein levels were not significantly different from the level of muscle β1D-integrin expressed by the wild-type cells under static conditions on day 4 (Fig. 4), and no detached cells were observed. Multinucleated myotubes were observed after 4 days; however, the efficiency of myotube formation was lower in these cells than the frequency observed in wild-type cells (Fig. 4), perhaps due to a functional deficiency in the human β1D-integrin protein functioning in the murine background. In addition, the myotubes appeared wider and nuclei appeared more aggregated within cells as opposed to the linear alignment observed mostly in wild-type cells.

β1D-integrin regulates RhoA activity. The increase in cellular β1D-integrin protein on mechanical stretch is associated with enhanced cytoskeletal structure and myotube formation. If β1D-integrin is required for optimal cytoskeletal structure, suppressing its expression should result in a less optimal cytoskeleton. Wild-type cells were used as controls because we did not observe any significant difference in the protein ex-

Fig. 2. Stretch activates β1D-integrin, focal adhesion kinase (FAK) phosphorylation at Tyr 379, and RhoA activity. Protein levels of total FAK (A), FAK phosphorylation (pFAK397; B), total RhoA (C), and RhoA activity (D) in response to stretch. E: decreases of FAK phosphorylation level from day 2 to day 4 were observed in static or stretched cells. Differentiating C2C12 cells were either left unstretched or subjected to stretch for up to 4 days. Total cell extracts were obtained at 48 h and 96 h. Protein expression was analyzed by Western blotting and normalized by the corresponding protein levels of unstretched cells on day 2. For all cases, the data were normalized to static cells 2 days after onset of differentiation. Values represent means (SD), and n = 3 or 4 separate experiments. *P < 0.05, **P < 0.01, ***P < 0.001 compared with static group.
pression levels of interest, compared with negative control cells under the same conditions (Figs. 5, C and D, and 6, A and B). As demonstrated in Fig. 5A, rhodamine phalloidin staining in β1D-integrin-repressed cells revealed that stress fibers were only localized to the sarcolemmal membrane and not widely dispersed throughout the cell, as is the case in control cells. Furthermore, there was an overall loss of stress fibers in the cytoplasm of β1D-integrin-deficient cells (compare Fig. 1A). These qualitative observations suggest that β1D-integrin is a key element of maintaining the cytoskeletal architecture.

β1D-integrin enhances cell contractility by promoting stress fiber formation (3). RhoA regulates actin polymerization and focal adhesion assembly (17). To further elucidate the functional relationship between β1D-integrin and RhoA, we inhib...

Fig. 3. Suppression of β1D-integrin inhibits differentiation in C2C12 cells. A.1: morphological change in short hairpin small interfering (si)RNA (shRNA)-treated cells compared with cells stably transfected with vector containing nonspecific scrambled shRNA sequence (shControl). A.2: repression efficiency of β1D-integrin protein levels in stably transfected cells relative to negative control (shControl) cells. Western blot analysis of cell extracts of C2C12 cells treated with shControl (lanes 1, 3, β1D-integrin RNA interference (lanes 2, 4) with the antibody against β1D-integrin. A.3: β1D-integrin protein levels in wild-type (WT) cells (lanes 1, 3) and shControl cells (lanes 2, 4). B: immunostaining of β1D-integrin in differentiating C2C12 cells treated with shRNA under static or stretched conditions on days 2 and 4. C: repression of β1D-integrin expression induces cell growth arrest in differentiating C2C12 cells. β1D-integrin-repressed cells were labeled and stained with bromodeoxyuridine (BrdU) under static or stretch conditions on days 2 and 4, compared with the same conditions used in wild-type and shControl cells. *P < 0.05; **P < 0.01; ***P < 0.001.
ited RhoA activity by introducing the RhoA inhibitor C3 transferase into the cells. As expected, inhibition of RhoA activity in wild-type cells impeded the fusion of myotubes in both unstretched and stretched cells (Fig. 5B). That suppression of \( \beta_{1D} \)-integrin expression and RhoA had similar effects on cytoskeleton structure, which suggests that the effects of \( \beta_{1D} \)-integrin are, at least in part, mediated through its regulation of RhoA activity.

Since inhibiting \( \beta_{1D} \)-integrin expression and RhoA activity had similar effects, we further hypothesized that the stretch-associated effects of \( \beta_{1D} \)-integrin are mediated through RhoA activation. No significant difference was found in total RhoA in control or \( \beta_{1D} \)-integrin-deficient cells with or without stretch (Fig. 5C). Repression of \( \beta_{1D} \)-integrin expression abolished stretch-induced increases in RhoA activity observed in wild-type cells, suggesting that the upregulation of RhoA-GTP under stretch conditions was mediated by \( \beta_{1D} \)-integrin (Fig. 5D). Furthermore, repression of \( \beta_{1D} \)-integrin expression resulted in a further decrease in RhoA-GTP activity in unstretched cells, compared with the wild-type \( \beta_{1D} \)-integrin-expressing cells. Under stretch conditions, RhoA activity in \( \beta_{1D} \)-integrin expression-deficient cells slightly increased, but it was below the level of unstretched wild-type cells and it was considerably below levels observed in stretched wild-type cells. The reduction of RhoA activity in \( \beta_{1D} \)-integrin-deficient cells was recovered by reexpression of human \( \beta_{1D} \)-integrin (data not shown). These results further corroborate our hypothesis that \( \beta_{1D} \)-integrin regulates RhoA activity under static and stretch conditions. However, it is feasible that another pathway [e.g., N-cadherin (8)] may exist to activate RhoA activity in response to stretch.

**Effects of repression of \( \beta_{1D} \)-integrin on FAK phosphorylation, NO production, and cell elasticity.** We next examined the effect of stretch on FAK phosphorylation in \( \beta_{1D} \)-integrin-deficient cells. Compared with the same condition for wild-type cells, total FAK was not significantly different (Fig. 6A). Cyclic mechanical stretch led to increases in FAK phosphorylation in wild-type cells, but stretch did not cause any change in FAK phosphorylation in \( \beta_{1D} \)-integrin-deficient cells (Fig. 6B) compared with the levels in wild-type cells under static conditions, suggesting that FAK is a downstream factor in a \( \beta_{1D} \)-integrin-mediated mechanical transduction pathway. No significant difference was found in the protein levels of total FAK and FAK phosphorylation in wild-type cells, relative
to negative control cells (cells transfected with scrambled siRNA) under the same conditions (Fig. 6, A and B).

Since NO release affects skeletal muscle differentiation (28, 39) and is regulated by focal adhesion stability (42), we hypothesized that the observed effect of β1D-integrin blockade was mediated, in part, by a block in the increase in NO. Cyclic stretch enhanced NO release (Fig. 6C) in a manner which is consistent with our previous study (47) and other reports (see, e.g., Ref. 41). Repression of β1D-integrin completely abolished the expected stretch-induced augmentation of NO production in that NO levels were not significantly different from the levels in unstretched cells. The rescue of human β1D-integrin expression in β1D-integrin-deficient cells resulted in expected increases in NO production (data not shown).

NO mediates the level of focal adhesion proteins and RhoA activity, thereby affecting the mechanical properties of the cells (47, 48). We observed that cyclic stretch increased the apparent elastic modulus (E_app) on days 2 and 4, which corresponded to increases in β1D-integrin protein and an enhanced actin-cytoskeletal structure (Fig. 1). When the expression of β1D-integrin was repressed, E_app further decreased in cells under either static or stretched conditions on day 4. The reduction in cell elasticity with repression of β1D-integrin suggests that β1D-integrin is critical for maintaining cytoskeletal integrity in differentiating skeletal myocytes (Fig. 6D).

**DISCUSSION**

Myogenic differentiation is a well-orchestrated process characterized by coordinated exit from the cell cycle, induction of differentiation by expression of muscle-specific genes, and the subsequent physical fusion of mononucleated cells into multinucleated syncytia, commonly referred to in the field as myotubes. Recent studies have shown that the β1D splice
variant of the β1-integrin, an isoform predominantly expressed in striated muscle, may play a pivotal role in myodifferentiation (2, 5, 35). For instance, β1-integrin knock-in mice exhibit impaired primary myogenesis, a process that requires cellular proliferation, with considerable reduction of muscle mass (5). Overexpression of either β1A- or β1D-integrin attenuates mature muscle cell differentiation and myotube formation during secondary myogenesis, suggesting a precise developmental regulation of these β1-integrin splice variants during skeletal muscle development (5). However, β1D-integrin does not act in C2C12 myoblasts through activation of MAPK (2). Engagement of β1D-integrin is required to alter SRF in a RhoA-dependent manner in cardiac myocytes (46). These findings led us to hypothesize that β1D-integrin controls skeletal myocyte differentiation in a precisely regulated manner that involves RhoA activation and the downstream phosphorylation of FAK and release of NO. We observed that, indeed, β1D-integrin appeared to regulate skeletal myocyte differentiation through RhoA activity, and, furthermore, in response to mechanical stretch, β1D-integrin activated the downstream mediators RhoA and FAK. Repression of β1D-integrin expression led to the remodeling of cytoskeleton and inhibited myotube formation. Reexpression of β1D-integrin by transfection of a human β1D-integrin in β1D-integrin-deficient cell lines restored myoblast fusion.

β1D-integrin is localized to costameres and myotendinous and neuromuscular junctions (44), which are the primary sites to sense and convey environmental cues from extracellular matrix to translate them into a series of biochemical signals. Four days of cyclic stretch enhanced cytoskeletal architecture, indicated by cross-striation of α-actinin staining and organized actin stress fibers (Fig. 1 and Table 1). The enhanced cytoskeletal structure, induced by stretch, appears to be associated with increases in β1D-integrin (Fig. 1, C and D). This is consistent with a recent report that mechanical stretch induces myosin heavy chain expression and muscle differentiation (30). The myocyte differentiation induced in response to stretch may represent a cellular response to increased stresses on focal adhesion contacts. Increased levels of β1D-integrin and enhanced actin cytoskeleton structures in response to mechanical stretch may enhance the stability of muscle focal adhesive structures. The linkage between β1D-integrin and talin is high affinity, and expression of β1D-integrin in nonmuscle cells leads to augmented contractility and adhesion (3). Furthermore, β1D-integrin overexpression in embryonic stem cells resulted in similar observations (14).

β1D-integrin initiates downstream signaling cascades on stretching of cells. We observed that β1D-integrin was significantly upregulated in response to stretch, and this was associated with corresponding increases in RhoA activity and pFAK397 (Fig. 2, B and D). The significant increase of β1D-integrin in stretched cells on day 2 coincided with the onset of myotube formation (Fig. 1, C and D), implying that the expression of β1D-integrin is required for myocyte fusion to
occurred. Reexpression of a human β1D-integrin into the murine environment partially restored myocyte fusion (Fig. 4), consistent with the role of β1D-integrin in determining the transition from proliferation to fusion during myogenic differentiation. We observed that β1D-integrin mediated FAK phosphorylation and RhoA activity in response to stretch, while inhibition of β1D-integrin expression repressed stretch-induced increases in FAK Tyr397 and RhoA activity (Fig. 6). Together, our results suggest that FAK and RhoA are involved in β1D-integrin-mediated mechanotransduction.

The increased levels of RhoA activity and FAK phosphorylation enhance actin cytoskeleton by inducing stress fiber formation, focal adhesion assembly, and, subsequently, the assembly of mature sarcomeres (9, 31). Initially, the activation of RhoA in myoblasts on stretch may accelerate myofusion by mediating the expression of important muscle regulators, MyoD, Mrf5, and other myogenic genes, via the transcriptional regulator SRF (7, 40). Our results are in agreement with these studies showing that inhibition of active RhoA by induction of C3 transferase into the cells impaired myodifferentiation and of RhoA in myoblasts on stretch may accelerate myofusion by mediation of important muscle regulators, MyoD, Mrf5, and other myogenic genes, via the transcriptional regulator SRF (7, 40). Our results are in agreement with these studies showing that inhibition of active RhoA by induction of C3 transferase into the cells impaired myodifferentiation and actin fiber formation and RhoA was positively responsive to mechanical stimuli in vivo (Figs. 2D and 5, B and D; Refs. 25, 46). Our data suggest that RhoA activity is at least partially mediated by β1D-integrin. This is supported by the observation that expression of β1D-integrin in β1D-integrin-deficient cells results in activation of RhoA and reorganization of actin cytoskeleton (15). RhoA activity was present at a reduced level in β1D-integrin-deficient cells with and without stretch, indicating an alternate pathway for activation of RhoA GTPases (Fig. 6C). However, this level of RhoA activity was not sufficient to induce differentiation.

Similarly, FAK is also an important component in β1D-integrin-mediated signaling pathway. A dominant mutant negative FAK reduced the proliferation induced by cyclic stretch (21), while overexpression of dominant-negative FAK led to cell cycle-arrested cells and consequently, few cells fused into myotubes (11, 21). We observed that repression of β1D-integrin did not significantly alter FAK autophosphorylation, relative to wild-type cells, whereas RhoA activity was reduced, suggesting that FAK can be phosphorylated by other proteins than RhoA and a minimum level of phosphorylation of FAK is associated with cell attachment. This is supported by the findings that FAK can be phosphorylated at the site of Tyr 397 independent of integrin clustering (23, 27).

We previously observed (47) that endogenous NO release modulates mechanical properties of differentiating skeletal myocytes through focal adhesion proteins and inhibition of protein degradation. A recent study by Zuckerbraun et al. (48) reports that NO influences RhoA activity in smooth muscle cells. In the present study, we observed that blocking β1D-integrin expression led to decreased NOS and RhoA activity, which resulted in weakened focal adhesions and compromised actin cytoskeleton in these cells. These changes in the cytoskeletal structure were reflected in the mechanical properties of the cells. We observed decreased transverse elastic modulus in β1D-integrin-deficient C2C12 cells (Fig. 6D). Together, our findings support a model in which muscle cells respond to mechanical stress in a coordinated manner (Fig. 7). This model involves roles for β1D-integrin, FAK, RhoA, and nNOS in processes involved in myocyte fusion, muscle differentiation, and response to mechanical stimuli by reinforcing the cytoskeleton and increasing muscle stiffness. This model is supported by the observation of others in studies of skeletal myogenesis and responses of myocytes to mechanical stimuli (4, 11, 20, 40). In this model, the switch of expression of β1D-integrin from β1A-integrin is induced on stretch. After initial integrin ligation, β1D-integrins attach to the actin cytoskeleton through high-affinity binding to talin. The clustering of integrin induces recruitment of FAK and subsequently FAK tyrosine phosphorylation. Also, the activation of the RhoA GTPases by β1D-integrin further promotes integrin clustering and FAK phosphorylation. nNOS is another important protein in the proposed model, in which it exerts its effect by producing the signaling molecule NO. nNOS is localized at the sarcolemma and directly associated with dystrophin. The membrane-bound signaling protein senses the mechanical cues and subsequently releases NO to regulate various aspects of muscle function in skeletal muscle.
regulating RhoA, β1D-integrin and nNOS may interact with one another.

In summary, we have observed an important role for β1D-integrin in a number of processes important for myogenesis, muscle differentiation, and response to mechanical stimuli that involve downstream functions for activated RhoA, phosphorylated PAK, and nNOS-produced NO. As these factors are almost certainly involved in other important and cellular interactions in muscle and other proteins are involved in the processes studied herein, the regulatory network mediating myogenesis in response to mechanical stress likely is even more complex and worthy of further study.

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